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Stability of Amylase Crude Powder from *Aspergillus awamori* KT-11 at Different Storage Temperatures

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***Abstract*— Amylase is one kind of biotechnology product that has been used in many sector industries. Production of amylase powder from *Aspergillus awamori* KT-11 has been optimized successfully in the previous study. However, the stability of its activity, which is crucial in industrial application, has not yet been well studied. This study aimed to investigate the stability of amylase crude powder activity from *A. awamori* KT-11 at different storage temperatures. Amylase crude powder was stored at 4ºC and 25ºC. For the stability test, the crude powder with optimum storage temperature was diluted with phosphate buffer pH 4.8 and stored at 4ºC, 25ºC, and -20ºC. The results showed that the activity of amylase crude powder was stable at 25 ºC after nine months of storage with total activity of 2807 U/mL. On the other hand, the amylase extract liquid enzyme shows better activity when stored at 4 ºC (with total activity 1000 U/mL after four-week storage time) than stored at temperatures 25ºC or -20 ºC. The findings also showed that crude amylase liquid extracts are not stored in a negative temperature environment. This is beneficial to enterprises because it lowers the cost of obtaining negative temperatures and saves energy.**

***Keywords*— Enzyme stability; *Aspergillus awamori* KT-11; storage temperature; extract liquid enzyme.**

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I. INTRODUCTION

Indonesia is one of the agricultural countries that produce roots and tubers, one of which is cassava. Cassava (*Manihot esculenta* Crantz) is one of the important commodity crops in Indonesia that can play a vital role in food security and sustainability [1]. Cassava productions in Indonesia reach 25 million tons in 2019. Some parts of the cassava plant, such as its storage root and leaves, are commonly utilized for human consumption. The cassava storage root is widely used as a staple food or snack [2]. In addition to a food crop, cassava is also used as raw material for various industries such as *biocomposites* [3], *bioethanol* [4], fermentable sugar [5]. Usually, other parts of cassava storage root, cassava peels, are used for animal feed. However, in terms of bioproducts, development, not only its starchy root but the waste or residues generated during cassava processing such as peels, stems, or wastewater identified as toxic to the environment can also be converted into high value bioproduct [3], [5]. As most cassava waste still contains starch and cellulose components, it is necessary to process this component into a simple fermentable sugar through hydrolytic and cellulolytic enzymes such as amylase and cellulase. *Hydrolytic enzymes* account for a notable share of the world enzyme market and thus are of great significance from industrial and biotechnological perspectives. The utilization of cassava peel as a substrate for the production of amylase also has been done by several studies [6], [7].

Amylase has been used in many industries such as in textile industries for smoothening the fabric [8], baking industries for improving the texture and taste from bread, laundry industries in detergent composition [9], beverage industries to get high sugar concentration in fruit juice [10], and paper industries for degrading starch [11]. Regarding *Amylase* productions, other studies have performed several reports to optimize amylase production to seek the best amylase activity for industrial applications. Some parameters such as microbial sources, temperature, substrate, fermentation time, *pH*, *nitrogen* sources, and moisture have been found to affect amylase production [11], [12].

Recently, the use of enzymes in various industrial sectors is increasing. Therefore, it is very important to have several properties such as the biophysical properties of enzymes, the catalytic efficiency of enzymes with substrates, specific substrates, and enzyme stability optimized for industrial purposes [13]. The stability of enzyme activity is one of the important properties in enzyme-based industries [14]. Despite many uses, the practical industrial applications of amylases in different processes are often confined by their short lifetime, high sensibility to the environmental conditions, undesirable operational storage stability, high production costs resulting from the difficulties in recovery and reusability [11]-[14].

Our previous study has focused on optimizing the production of amylase powder from A. awamori KT-11 in solid-state fermentation [6]. However, the stability of the amylase powder has not yet been studied. Therefore, in this study, we researched the stability of amylase activity from *A. awamori* KT-11 based on a storage method using different temperatures.

II. Materials and Methods

All experiments were performed in triplicate, and the values are given as mean ± standard deviation (SD). All chemicals were of analytical grade. The cassava peels were obtained from cassava germ plasma belongs to Research Center for Biotechnology-LIPI. The research flow chart is presented in Fig. 1.

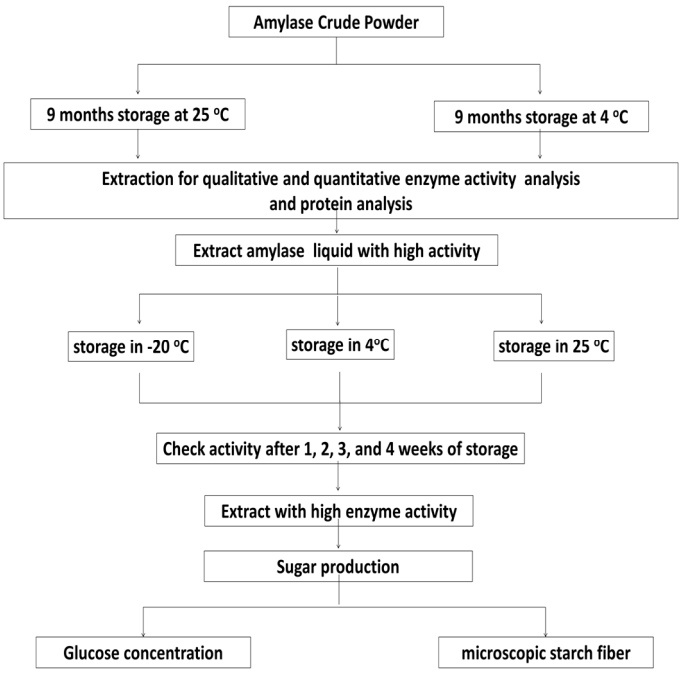


Fig. 1 Research flow stability of amylase crude powder from *A. awamori* KT-11

A. Isolate Preparation

The fungi *Aspergillus awamori* KT-11 isolate is provided by Research Center for Biotechnology. Recovery of this isolate from 20% (w/w) glycerol stock was performed using a potato dextrose agar (PDA) plate. Culture maintenance was conducted by streaking on Potato Dextrose Agar (PDA) medium and incubated at room temperature for 5 (five) days. The *A. awamori* KT-11 spores were dissolved in 10 mL of distilled water. The spore's solution was ready to use as an inoculum for enzyme production.

B. Enzyme production

Cassava peels were used as the main solid component in the medium composition for enzyme production. Around 500g of cassava peels were chopped, then 50 mL solutions contained 5g K_2HPO_4, 5g KH_2PO_4, 12.5g $(NH_4)_2SO_4$ were added and mixed evenly. Subsequently, the media was sterilized at 121°C for 15 minutes and then allowed to room temperature. The production of amylase crude powder was conducted by inoculating spores of *A. awamori* KT-11 from PDA into the medium production. The culture was then incubated in solid-state fermentation for five days at room temperature, periodically stirring once a day. Afterward, the culture containing the amylase crude powder was dried at 50-60°C using dry-oven (Sanyo MOV 212S, Japan) for two days, and the dried sample was ground into a fine powder (200 mesh) [6]. Amylase crude powder was placed in an airtight plastic bag and kept in a desiccator cabinet (Krisbow-dry cabinet, China) for moisture-proof closure at room temperature until analysis.

C. Enzyme Stability Test

The stability tests were conducted on amylase crude powder and amylase extract liquid enzymes (Fig. 1). For the amylase as crude powder enzyme, after nine months stored, the stability tests were carried out at two different storage temperatures, which are 4°C and 25°C. Meanwhile, for the amylase as a liquid extract, the stability tests were carried out at three different storage temperatures: 4°C, 25°C, and -20°C. The enzyme activity was analyzed by dissolving amylase crude powder into phosphate buffer pH 4.8 (1:9), then shaking the mixture at 120 rpm, for 20 minutes at room temperature using an incubator shaker (Taitec BR-43FL, Japan). Subsequently, the filtrate was separated from their solid by centrifugation at 10.000 rpm for 15 minutes at 4°C [6]. A portion of enzyme extracts was tested for their activity by qualitative and quantitative analysis, and the rest were stored for the stability test on storage at -20, 4, and 25°C (Fig. 1).

D. Analysis Qualitative of Amylase Activity

The qualitative analysis was carried out to determine the initial activity of amylase crude powder. About 5µL of crude extracts liquid amylase was dropped onto agar plates containing 1% starch as substrate. The agar plate was incubated at 37°C for 18 hours. Afterward, Lugol's iodine was added into the agar plate. The plates were incubated at room temperature for 1 (one) hour, and the clear zone formation was observed. Transparent circles from the blue background of the starch-iodine compound indicated the amylase activity [15].

E. Analysis Quantitative of Amylase Activity

A 10% soluble starch was used for amylase activity analysis as a substrate. About 400 µL substrate was added into 100 µL of the crude extracts liquid amylase and homogenized by vortexes. Afterward the sample was incubated at 60°C for 60 minutes and subsequently heat up in boiling water for 1 minute to stop the amylase activity. The concentration of sugar obtained from hydrolysis was analyzed by Somogyi

Nelson method [16]. One-unit amylase activity was defined as the amount of amylase which released 1 mol glucose.

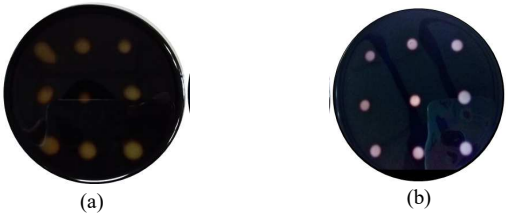
F. Sugar Production

Amylase crude powder with the highest activity was tested for its ability to produce sugar from cassava starch. The sugar productions were performed in a 1-L flask liquid medium containing 30% (w/v) of cassava starch and 10% crude amylase liquid extract. The sample was incubated at 60°C for 72 hours for fermentation and stirred twice a day to keep homogeneous. At the end of fermentation, the supernatant was collected from cassava starch sediment by centrifugation at 10.000 rpm for 15 minutes at 4°C. Afterward, the supernatant was heated at 100°C to stop the enzymatic process. The sugars content on the supernatant was analyzed using High-Performance Liquid Chromatography (HPLC) (Shimadzu LC-20AB, Kyoto, Japan). A Bio-Rad (Hercules, CA) *Aminex HPX87H* column (300-7.8 mm) was used for sugar analysis at 60°C using a refractive index detector RID-10A. Sulfuric acid (H_2SO_4) buffer at concentration 0.01 N was used as mobile phase at a flow rate of 0.6 mL/min [17]. All samples were centrifuged to remove the cell mass and other water-insoluble substances and then filtered through a 0.22 µm filter before analysis. The pellet obtained from centrifugation was analyzed for the starch fibers characterization by Scanning Electron Microscopy (SEM) JEOL JSM-IT 200 (Jeol Ltd., Japan) with 1000x magnification.

III. Results and Discussion

Enzyme stability is one of the enzyme properties that can be used as an indicator in the enzyme production processes. Enzyme stability is influenced by certain conditions such as temperature and storage time. In this study, enzyme production was performed through solid-state fermentation (SSF). SSF method has several advantages: higher concentration, higher productivity, and higher product stability [18].

The qualitative assessment of storage stability was conducted to check the amylase crude powder activity which has been stored for nine months following the production step. As shown in Fig 2, the amylase crude powder consistently had an activity after 9-month of storage, either at 4°C or 25°C. A clear zone on the agar medium indicates that amylase was able to hydrolyze starch in the media. Meanwhile, the unhydrolyzed starch will accumulate the blackish-blue color when tested using Lugol's iodine [15]. The clear zone from the amylase crude powder at 25°C was approximately 1 mm larger than that obtained at 4°C. This clear zone size indicates the amylase activity, where the larger clear zone shows higher amylase activity. The diameter of the clear zone is relatively proportional to amylase activity [19].



(a) (b)

Fig. 2 Analysis qualitative amylase activity after 9-month storage (a: 25°C, b: 4°C)

The quantitative analysis of amylase activity was carried out by Somogyi Nelson [16]. Figure 3 shows the amylase activity in crude powder after 9 (nine) months of storage. As shown in Fig 3, the activity of amylase crude powder from storage at 25°C was significantly higher than at 4°C. The activity was achieved at 2807 U/mL and 80 U/mL, for storage at 25°C and 4°C, respectively. This finding agrees with the previous study by Moura *et al.* [20], which found that the enzyme powder from SSF process can be stored at 25°C and does not need to store at low or negative temperatures. The enzyme powder from SSF is more stable for long-term storage, which can improve its economic value, and viability process technology utilization of enzyme [21]. Based on the qualitative and quantitative analysis, amylase crude powder stored at 25 °C showed higher activity than 4°C. Therefore, the amylase crude powder stored at room temperature was used for further analysis in this study.

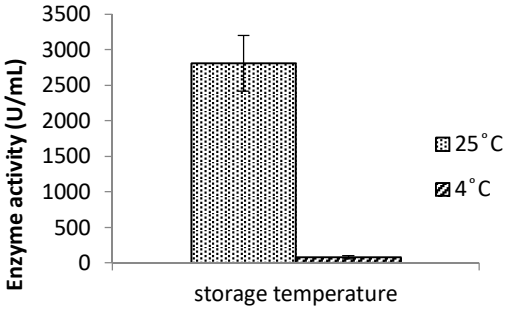


Fig. 3 Activity of amylase crude powder after nine months storage

The storage stability of crude amylase liquid extract was also evaluated at three different storage temperatures of 25°C, 4°C, and -20°C. As shown in Fig 4, the initial activity of crude amylase liquid extract stored at 25°C was recorded around 2807 U/mL and decreased by time after 4 weeks of storage to around 1000 U/mL. The percentage degradation activity of crude extracts liquid amylase stored at 25°C for four weeks was recorded every week based on Fig 4.

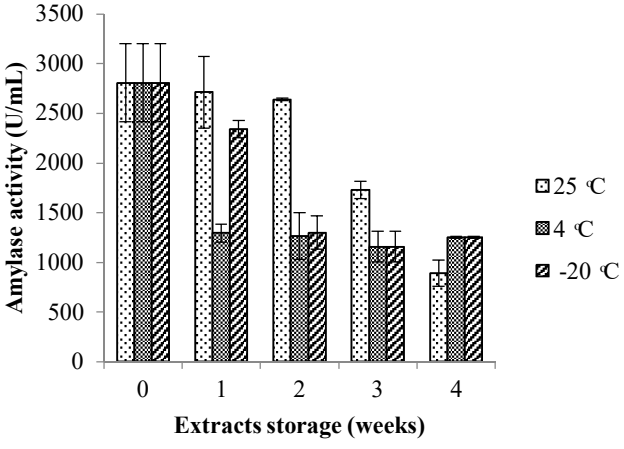


Fig. 4 Activity of crude amylase liquid extracts

The first week remained 96% from the initial activity, followed by 94%, 61%, and 31% for 2nd, 3rd, and 4th weeks, respectively. In the case of crude extracts liquid amylase stored at 4°C, the percentage of activity was dramatically

decreased in the first week (remain 46% from the initial activity) and tend to be constant in the second to fourth week of storage time (at 45%-41%). A similar pattern was also obtained for the crude amylase liquid extract stored at -20°C. The amylase activity was remained 83% in the first week of storage, then stable at 46-41% until the fourth week of storage time.

The decreased in amylase activity is more likely due to protein denaturation, which affected by temperature fluctuations. Some chemicals that are often used as protein protectors to keep amylase activity are remaining stable from temperature fluctuations include sugar alcohol (glycerol, lactose, mannitol, sorbitol, and sucrose), bovine serum albumin (BSA), human albumin, gelatin, polyethylene glycol (PEG), surfactants, and micelles [14]. Furthermore, some enzymes such as amylase, lipase, trypsin and leucine–alanine peptidase are not recommended to be stored in homogenate form at -20°C because it will reduce its activity [22].

In this study, the crude extracts liquid amylase protein content during four weeks of storage was also compared with the protein content in the initial extracts. Fig. 5 shows the protein concentrations in the crude extracts liquid amylase. As shown in Fig. 5, the initial extracts' protein content was higher than that obtained during 4 weeks of storage. Protein content was decreased by up to 20-40% from the initial at all temperature storage. Therefore, it is not recommended to store enzyme extracts for a long period. This result shows a positive correlation between amylase activity and protein denaturation. The protein damage might appear due to the oxidation reactions affected by *nitrogen* or sugars [22].

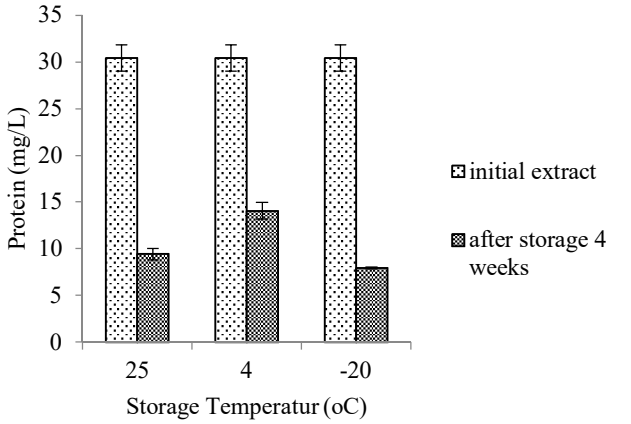
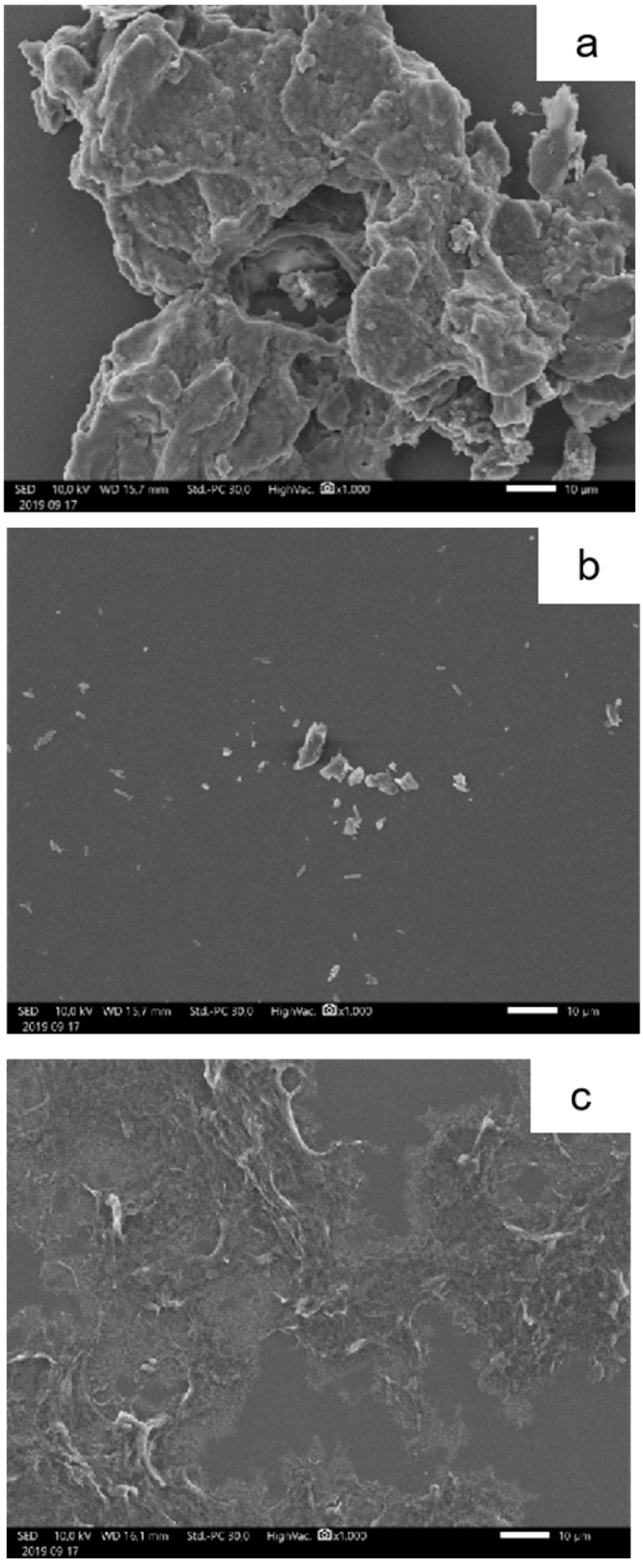


Fig. 5 Protein concentrations in crude amylase liquid extract

The amylase activity was also evaluated for a 9-month storage time in their ability to hydrolyze starch. Sugars analysis performed by HPLC showed that the hydrolysis products consist of glucose, maltose, and fructose. The concentrations of sugars were gradually increased day by day. The glucose concentration was 80.25 g/L, maltose was 68.88 g/L, and fructose was not yet detected at 24 hours of incubation while there was an increase in glucose, maltose and fructose concentration 89.98 g/L, 64.54 g/L, and 0.86 g/L, respectively after 72 hours of incubation. The results obtained illustrate that the glycosidic bonds cutted-off by amylase in a row will release glucose from the end of the starch backbone without breaking the chain [23].

Morphological observation of cassava starch before and after hydrolysis by scanning electron microscopy (SEM) photographs are shown in Fig 6. The unhydrolyzed starch by amylase is consistently in the shape of large lumps (Fig 6a). The lump structure is due to the previous gelatinization process due to 8.5% amylose of cassava starch being swelled after heat treatment at 60°C [24]. After 24 hours of incubation with amylase, the starch swell appears to be getting smaller than before (Fig 6b).



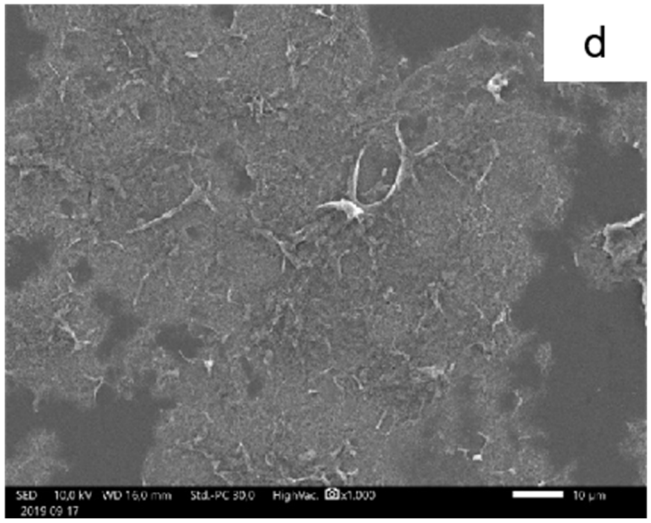


Fig. 6 Scanning electron micrograph (SEM) with 1000x magnification from starch fibers that are hydrolyzed by amylase from *A. awamori* KT-11 (a: 0 hours; b: 24 hours; c: 48 hours; d: 72 hours).

As the hydrolysis reaction time increases, the starch lumps decrease, the starch gel lumps change to form liquid sugar (Fig 6c-6d). The increase in liquid volume is due to increased water molecules due to the hydrolysis of starch polymer into sugar monomer [25]. This indicates that the amylase produced by *A. awamori* could hydrolyze starch lumps and form other simple sugars [26]–[28].

IV. Conclusion

From this study, it can be concluded that the amylase crude powder from *A. awamori* KT-11 stored for 9 months still has hydrolytic activity. Amylase crude powders from *A. awamori* KT-11 is better stored at 25 °C rather than 4 °C. While the crude extracts, liquid amylase should be used immediately because the activity decreases with increased storage time. Crude amylase liquid extracts were more stable at 4°C than 25 °C and -20°C storage temperature. The results also indicated that the crude amylase liquid extracts do not need to be kept at negative temperature. This is advantageous for industries since it reduces the cost of obtaining negative temperature and energy savings.

Acknowledgment

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